



UNIVERSITÀ  
DEGLI STUDI  
FIRENZE

## FLORE

# Repository istituzionale dell'Università degli Studi di Firenze

### **The vitamin D analogue BXL-628 inhibits growth factor-stimulated proliferation and invasion of DU145 prostate cancer cells.**

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

*Original Citation:*

The vitamin D analogue BXL-628 inhibits growth factor-stimulated proliferation and invasion of DU145 prostate cancer cells / S. MARCHIANI; L. BONACCORSI; P. FERRUZZI; C. CRESCIOLI; M. MURATORI; L. ADORINI; G. FORTI; M. MAGGI; E. BALDI. - In: JOURNAL OF CANCER RESEARCH AND CLINICAL ONCOLOGY. - ISSN 0171-5216. - STAMPA. - 132:(2006), pp. 408-416. [10.1007/s00432-006-0086-8]

*Availability:*

This version is available at: 2158/310398 since: 2019-07-23T17:12:14Z

*Published version:*

DOI: 10.1007/s00432-006-0086-8

*Terms of use:*

Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (<https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf>)

*Publisher copyright claim:*

(Article begins on next page)

Sara Marchiani · Lorella Bonaccorsi  
Pietro Ferruzzi · Clara Crescioli · Monica Muratori  
Luciano Adorini · Gianni Forti · Mario Maggi  
Elisabetta Baldi

## The vitamin D analogue BXL-628 inhibits growth factor-stimulated proliferation and invasion of DU145 prostate cancer cells

Received: 23 May 2005 / Accepted: 9 January 2006 / Published online: 17 February 2006  
© Springer-Verlag 2006

**Abstract Purpose:** Suppression of the invasive phenotype is essential in developing new therapeutic tools to treat advanced prostate cancer (PC) indicating that androgen-independent prostate cancer (AI-PC) is characterized by increased metastatic potential. In the present study, we have investigated the effect of the nonhypercalcemic vitamin D analogue BXL-628 on proliferation and invasive properties of the human PC cell line DU145. In particular, the effect of the analogue was tested following stimulation with a potent growth factor, keratinocyte growth factor (KGF), which stimulates both proliferation and invasion of these cells. We have also evaluated the effect of the analogue on KGF stimulation of PI3K/AKT signaling pathway. **Methods:** Cell proliferation was determined by cell counting. Invasion through Matrigel was evaluated using Boyden chambers. PI3K activity was measured by immunokinase assay and AKT phosphorylation was evaluated by western blot analysis. Keratinocyte growth factor receptor (KGFR) autotransphosphorylation was evaluated by western blot after immunoprecipitation of the receptor. **Results:** BXL-628 is able to inhibit both proliferation and invasion of DU145 cells in basal conditions and in response to KGF. Following stimulation with KGF, the inhibition is due to suppression of

PI3K/AKT activation, both achieved following a brief (5 min) incubation with the analogue. This effect on KGFR autophosphorylation was still present when cells were treated with the  $\alpha$ -amanitin, an inhibitor of RNA transcription, indicating a rapid, nongenomic effect. **Conclusions:** Our results demonstrate that the vitamin D analogue BXL-628 is able to suppress KGF-induced proliferation and invasion of AI-PC cells in vitro, prospecting a possible use of the drug, which is currently in phase II clinical studies for benign prostatic hyperplasia, in the treatment of advanced prostate cancer.

**Keywords** BXL-628 · Keratinocyte growth factor · Invasion · Proliferation

**Abbreviations** AI-PC: Androgen-independent prostate cancer · KGF: Keratinocyte growth factor · KGFR: Keratinocyte growth factor receptor · PI3K: Phosphatidylinositol 3 kinase · VDR: Vitamin D receptor

### Introduction

Prostate Cancer (PC) is one of the most common cancer and the second leading cause of death in American men (Gronberg 2003). In the advanced stages of the disease androgen ablation therapy represents a valuable tool for the treatment of these patients. However, in almost all patients androgen-independent (AI) clones of tumor cells develop after a year of treatment and at this stage no other efficacious therapies are available. The mechanisms responsible for transition to androgen independence are still unclear (So et al. 2005), however, a striking characteristics of androgen-independent prostate cancer (AI-PC) is related to its higher invasive potential compared to androgen-dependent stages (Chung et al. 2005). In vitro studies using available androgen-sensitive human PC cell line and androgen-insensitive human PC cell line indicate that, at least in part, higher

S. Marchiani · L. Bonaccorsi · M. Muratori · G. Forti  
M. Maggi · E. Baldi (✉)  
Department of Clinical Physiopathology, Andrology Unit,  
Center of Research, Transfer and High Education DeNothe,  
University of Florence, Viale Pieraccini 6, 50139 Firenze, Italy  
E-mail: e.baldi@dfc.unifi.it  
Tel.: +39-0554271486  
Fax: +39-0554271371

P. Ferruzzi · C. Crescioli  
Department of Clinical Physiopathology, Endocrinology Unit,  
Center of Research, Transfer and High Education DeNothe,  
University of Florence, Viale Pieraccini 6, 50139 Firenze, Italy

L. Adorini  
BIOXELL, Milan, Italy

invasiveness of AI-PC may be due to loss of regulation of genes involved in invasion (Baldi et al. 2003). Bone metastases have been reported to occur in 85–100% of patients with advanced PC. Thus, novel therapies to increase the chance and the quality of life in patients with advanced PC should aim at inhibition, besides proliferation, also the invasive ability of the tumors.

Based on their anti-proliferative, pro-apoptotic and pro-differentiative properties, vitamin D analogues have been extensively studied as possible treatments for cancer (Nagpal et al. 2005).

Several studies have focused on the role of calcitriol and its receptor, the vitamin D receptor (VDR), in PC (Peehl and Feldman 2004) and clinical trials have shown the capacity of calcitriol to inhibit prostate specific antigen (PSA) increase in PC patients (Trump et al. 2004). Polymorphisms in the VDR gene have been implicated as risk factors for PC development and progression (Habuchi et al. 2000) and the growth inhibitory effects of calcitriol and its analogues have been well characterized in PC cells (Nagpal et al. 2005). However, much less is known about the effect of these compounds on the invasive ability of PC cells, although calcitriol has been shown to reduce invasion in PC cells (Sung and Feldman 2000; Schwartz et al. 1997).

A major problem with the clinical use of calcitriol is its hypercalcemia-inducing capacity, prompting the search for less hypercalcemic analogues. The less hypercalcemic Vitamin D analogue BXL-353 shows a strong antiproliferative activity in PC cell lines and benign stromal cell in vitro, being effective at very low concentrations (Crescioli et al. 2000, 2002). This compound inhibits in vitro growth of both benign prostate hyperplasia (BPH) and PC cells by disrupting KGF-induced growth, decreasing bcl-2 over-expression and inducing apoptosis (Crescioli et al. 2002, 2003). Strikingly, the effect of the compound on KGF-induced growth is mediated by inhibition of KGF-induced KGF receptor autotransphosphorylation following a brief (5 min) treatment (Crescioli et al. 2002), indicating the involvement of a rapid, nongenomic mechanism of the vitamin D analogue on growth inhibition in PC.

In the present study, we have investigated the effect of a recently developed vitamin D analogue, BXL-628, on KGF-induced invasion and proliferation of the androgen-independent PC cell line DU145. Previous data from our laboratory demonstrated the capacity of this analogue to decrease prostate cell proliferation both in vitro, using primary cultures of human BPH cells and in vivo, showing inhibition of prostate growth in intact and castrated, testosterone-replaced, rats (Crescioli et al. 2004). Based on these data, BXL-628 is currently being tested in phase II trials for the treatment of benign prostate hyperplasia. Results in the present study examine the effects of BXL-628 on KGF-induced autotransphosphorylation of its receptor, and downstream activation of the PI3K/AKT pathway, in AI-PC cells.

## Materials and methods

### Materials

**Analogue** 1-*a*-fluoro-25-hydroxy-16,23*E*-diene-26,27-bishomo-20-epi-cholecalciferol (BXL-628) was provided by BioXell (Milan, Italy). Anti-KGFR polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antiphosphotyrosine PY20 antibody and [ $\gamma$ - $^{32}$ P]ATP were obtained from ICN (Costa Mesa, CA, USA). Keratinocyte growth factor (KGF) was obtained from Prepro Tech EC (London, England). LY294002 was from Calbiochem (California, USA). Phosphoinositides were from AV-ANTI POLAR-Lipids, Inc. (Alabaster, AL, USA). Protein A and Protein G-Sepharose were obtained from Amersham Pharmacia Biotech Italia (Cologno Monzese, Italy). Matrigel was from Becton Dickinson (Franklin Lakes, NJ, USA). Protein measurement Coomassie kit was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Annexin-V-Fluos staining kit was obtained from Roche Molecular Biochemicals (Milan, Italy). DMEM, antibiotics, and other not specified reagents were purchased from SIGMA Chemical Co. (St. Louis, MO, USA).

### Cell culture

Androgen independent human cell lines, DU145 and PC3, were obtained from American Tissue Culture Collection (Bethesda, Maryland, USA) and maintained, respectively, in DMEM and HAM-F12 Coon supplemented with 10% FBS, penicillin (100 UI/ml), streptomycin (10 mg/ml), and glutamine (2 mM).

### Cell proliferation assay

All proliferation tests were performed after 24 h of cell starvation in phenol red- and serum-free medium containing 0.1% BSA. After starvation, cells were incubated in the same medium as before, with or without specific stimuli. Thereafter, cells were trypsinized, and each experimental point was derived from counting in the hemocytometer and then averaging at least six different fields for each well as previously reported (Crescioli et al. 2003). Experiments were performed seeding  $4 \times 10^4$  cells onto 12-well plates in growth medium and incubated for 48 h with (1) increasing concentrations of BXL-628 ( $1 \times 10^{-12}$ ,  $1 \times 10^{-11}$ ,  $1 \times 10^{-10}$ ,  $1 \times 10^{-9}$ ,  $1 \times 10^{-8}$  M) with or without fixed concentration of KGF (10 ng/ml) or fixed concentration of bFGF (10 ng/ml); (2) fixed concentration of LY 294002 (10 nM) with or without KGF (10 ng/ml). In the same experiment each experimental point was repeated in triplicate and experiments were performed at least three times. Cell growth results are

expressed as the percentage of growth compared with their relative controls.

#### Invasion assay

Invasion assays were performed as described previously (Bonaccorsi et al. 2000, 2004a, b) according to Albini et al. (1987) using the Boyden chambers equipped with 8  $\mu$ m porosity polyvinylpyrrolidone-free polycarbonate filters (VWR International, Milan, Italy). A thin layer of Matrigel solution (50  $\mu$ g/ml) was overlaid on the upper surface of the filter and allowed to gel by incubating the filters at 37°C for 30 min. Cell ability to invade the substrate was assessed by using some different stimuli: KGF (10 ng/ml), in presence or in absence of the inhibitor, BXL-628 ( $1 \times 10^{-8}$  M). These molecules were added to the bottom well of the Boyden chambers. Cells ( $9.5 \times 10^4$ ) were then added to the top of the chambers and incubated for 24 h at 37°C. Migrated cells were quantitated by counting cells with a Zeiss microscope (Oberkochen, Germany) equipped with brightfield optics (40 $\times$  magnification). Results are expressed as the percentage of number of migrated cells per high-power field with respect to control.

#### Immunoprecipitation and Western blot analysis

Protein extraction and immunoprecipitation were performed as previously described (Bonaccorsi et al. 1997). Briefly, cells were scraped in PBS supplemented with 1 mM  $\text{Na}_3\text{VO}_4$ , centrifuged and resuspended in lysis buffer [20 mM Tris, pH 7.4, 150 mM NaCl, 0.25% NP-40, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM phenylmethyl-sulfonyl fluoride (PMSF)]. After protein measurement (Coomassie kit), aliquots of cell lysates containing equal amount of proteins (500  $\mu$ g) were incubated for 1 h with 30  $\mu$ l of Protein A (or Protein G)-Sepharose for preclearing. Precleared lysates were then incubated for 1 h using 5  $\mu$ g of specific anti-KGFR antibodies on ice followed by overnight incubation at 4°C with 30  $\mu$ l of Protein A (or Protein G)-Sepharose. The immunobeads were washed three times in lysis buffer and then resuspended in 10  $\mu$ l of 2 $\times$  Laemmli's reducing sample buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, 2.5% pyronin, and 200 mM dithiothreitol), boiled at 95°C for 5 min and loaded onto 8% polyacrylamide-bisacrylamide gels. After SDS-PAGE, proteins were transferred to nitrocellulose membrane (Sigma Co., St. Louis, MO, USA) and incubated with the specific primary antibodies for 2 h in 1% BM blocking (Roche, Milan, Italy) in TTBS solution (Tris-buffered saline containing 0.1% Tween 20, pH 7.4), washed and incubated with peroxidase-conjugated relative secondary antibodies (1:4,000) for 2 h. After washing, the blots were incubated with enhanced chemiluminescence (BM, Roche, Milan, Italy) detection reagent and exposed to film. After the first blotting, nitrocellulose membranes were stripped at

50°C for 30 min in stripping buffer (100 mM 2 $\beta$ -mercaptoethanol, 2% sodium dodecyl sulphate, 62.5 mM Tris-HCl pH 6.7) and re-probed with specific primary antibodies to detect different proteins.

#### Annexin-V binding assay

Annexin-V binding assay was used to detect translocation of membrane phosphatidylserine (PS) from the inner to the outer side of the plasma membrane, since the exposure of PS is considered an early sign of apoptosis (Kagan et al. 2000). The assay was performed by using the "Annexin-V-Fluos staining Kit" (Roche). Before treatment, cells ( $1 \times 10^6$ ) were kept in serum-free medium for at least 24 h, then cells were incubated for 8 h in the presence or absence of BXL-628 ( $1 \times 10^{-8}$  M), then cells ( $1 \times 10^6$ ) were washed, trypsinized, centrifuged. After two washes in PBS, cells were resuspended in 100  $\mu$ l of incubation buffer (supplied by manufacturer), 2  $\mu$ l of Annexin-V-Fluos labeling reagent (Ann-V-F, Annexin-V conjugated to fluorescein, supplied at the 200 $\times$  concentration by Roche), and 2  $\mu$ l of propidium iodide solution (PI, 30  $\mu$ g/ml in PBS) were added. After incubation (15 min in the dark at room temperature) samples were analyzed by flow cytometry. For each experimental set, two cell suspensions were prepared for instrumental setting and data analysis: (1) by omitting both Ann-V-F and PI staining (nonspecific fluorescence sample), and (2) by omitting only the PI staining (sample for compensation, see below). Ann-V-F green fluorescence and PI red fluorescence were revealed by using FL-1 and FL-2 detectors, respectively. Fluorescence compensation was set by acquiring sperm labeled with only Ann-V-F. For each sample 10,000 events were recorded at a flow rate of 200/300 cells/s. Debris was gated out by establishing a region around the population of interest, in the Forward Scatter/Side Scatter (FSC/SSC) dot plot. Quadrant setting was established in the FL-1/FL-2 dot plot corresponding to the autofluorescence sample by including more than 99% of total events in the lower left quadrant.

#### PI3K assay

PI3K activity was evaluated in vitro assay as previously described (Luconi et al. 2004). Briefly, cells were stimulated with KGF (5 min) in the presence or absence of BXL-628, scraped in PBS supplemented with 1 mM  $\text{Na}_3\text{VO}_4$ , centrifuged and extracted with lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 1% NP-40, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM PMSF). After measurement of proteins, the aliquots of cell extracts containing equivalent amount of proteins (500  $\mu$ g) were incubated for 1 h with 50  $\mu$ l of Protein G-Sepharose for preclearing. Precleared lysates were then incubated with an antiphosphotyrosine PY20 antibody overnight at 4°C with 50  $\mu$ l of Protein G-Sepharose as described above. The Sepharose beads were washed two

times with lysis buffer and twice with a 10 mM Tris-HCl (pH 7.4) containing 0.1 mM EGTA and 5 mM LiCl. After removal from the last wash, the beads were suspended in kinase buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA) containing 20  $\mu$ g of L- $\alpha$ -phosphatidylinositol, 25 mM MgCl<sub>2</sub>, and 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP, and incubated for 20 min at room temperature. The reaction was stopped by the addition of 60  $\mu$ l of HCl 6 M and 160  $\mu$ l of a mixture of chloroform and methanol (1:1). Lipids were then resolved by thin layer chromatography plates, TLC silica gel 60 (Merck Laborchimica, Florence, Italy), in chloroform, methanol, water, and ammonium hydroxide (60:47:11.3:2). Dried TLC sheets were developed by autoradiography. Quantifications of the bands were performed using a Kodak image analysis system.

### Statistical analysis

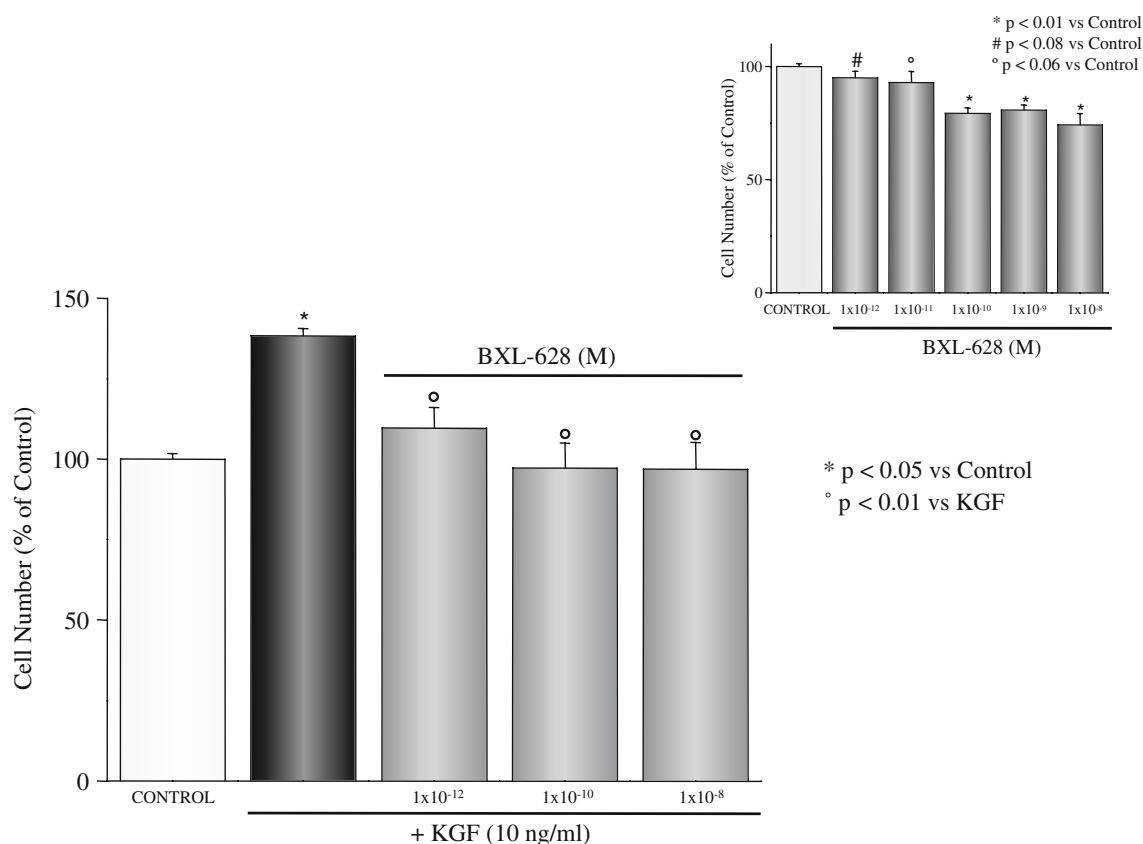
All the data are shown as mean  $\pm$  SEM of the indicated number of experiments. Statistical analysis was performed with ANOVA and Student's *t* test for unpaired and, when applicable, for paired data. IC<sub>50</sub> for dose-

response curves was calculated using the program ALLFIT.

## Results

### Inhibitory effects of BXL-628 on basal and KGF-mediated proliferation of DU145 cells

As shown in the inset of Fig. 1, treatment with the vitamin D analogue BXL-628 inhibits dose dependently DU145 cell proliferation with an IC<sub>50</sub> of  $22.1 \pm 19.1$  pM. Similar results are obtained when cell proliferation was assessed using the MTT assay (results not shown). As shown in Fig. 1, KGF stimulates DU145 cell proliferation at the concentration of 10 ng/ml. Treatment with BXL-628 completely and dose dependently inhibits proliferation stimulated by the growth factor (Fig. 1). Similar effects were also observed in the AI cell line PC3 [percentage number of cells:  $100 \pm 17$  control,  $121.3 \pm 13$  KGF (10 ng/ml),  $69.9 \pm 9.9$  KGF + BXL-628 ( $1 \times 10^{-8}$  M)], although, in line with previous work by our group (Crescioli et al. 2002), responsiveness of PC3 cells to KGF was lower with respect to DU145. To evaluate whether the inhibitory effects of BXL-628 were specific



**Fig. 1** Effect of BXL-628 on basal (*inset*) and KGF-stimulated proliferation of DU145 cells, determined by cell counting. Cells were treated for 48 h with increased concentrations of the analogue with or without fixed concentrations of KGF (10 ng/

ml). Each experimental point was determined in triplicate and experiments were performed at least three times. Results are expressed as the percentage of growth compared with their relative controls



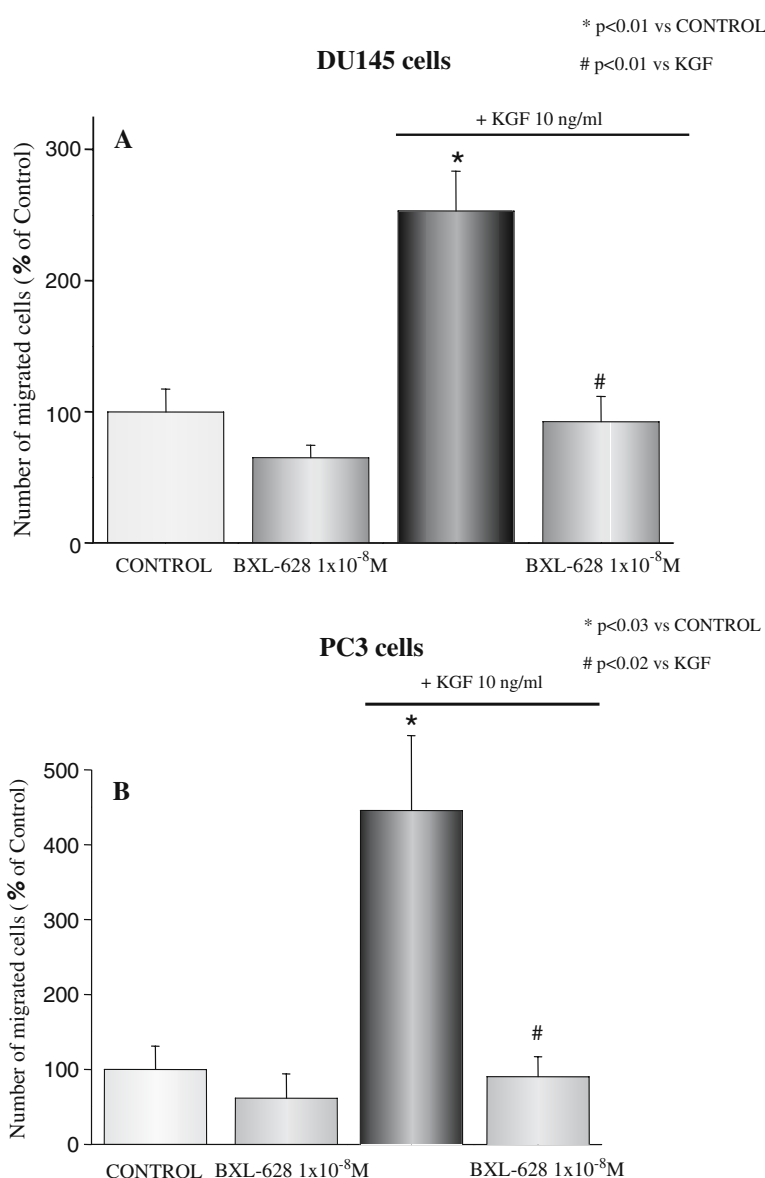
for KGF, we tested its effect on bFGF-stimulated proliferation of DU145 cells. Our results demonstrated an inhibitory effect of BXL-628 also on bFGF-mediated proliferation [percentage number of cells:  $100 \pm 8.6$  control,  $138 \pm 19.5$  bFGF (10 ng/ml),  $74.1 \pm 9.2$  bFGF + BXL-628 ( $1 \times 10^{-8}$  M)].

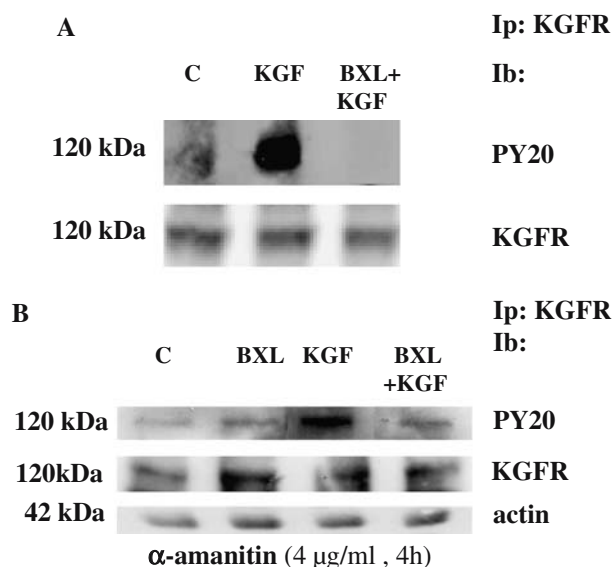
Previous studies from our group (Crescioli et al. 2000, 2002) indicate that vitamin D analogues exert in part their antiproliferative effects by inducing cell apoptosis. To evaluate whether the inhibitory effect of BXL-628 on DU145 proliferation was due to induction of apoptosis, we evaluated phosphatidylserine exposure (an early sign of cell apoptosis, for review see Kagan et al. 2000) in live cells by Annexin-V binding after 8-h incubation in the presence of the analogue ( $1 \times 10^{-8}$  M). We found that BXL-628 induces a significant increase of Annexin-V binding to the cells (percentage Annexin-V positive live cells:  $57 \pm 1.7$  control,  $62 \pm 1.2$  BXL-628,  $n=6$ ,  $P=0.017$ ).

## Effect of BXL-628 on KGF-induced Matrigel invasion of DU145 cells

Next, we investigated the effect of BXL-628 on KGF-stimulated Matrigel invasion. Previous studies investigating the effects of vitamin D analogues on cancer cell invasion and migration utilized long-term treatment protocols with at least 48 h cell preincubation before performing the invasion assay (Yudoh et al. 1999; Koli and Keski-Oja 2000; Schwartz et al. 1997). The inhibitory effect of KGF-induced KGFR autotransphosphorylation obtained after short-term (5 min) exposure to BXL-353 in BPH and PC cells (Crescioli et al. 2002) prompted us to evaluate the effect of BXL-628 on in vitro invasiveness of DU145 cells avoiding preincubation of the cells with the analogue, which was added directly to the bottom of Boyden chambers. As shown in Fig. 2 (panel a), the stimulatory effect of KGF on DU145 cell invasion is completely inhibited by the vitamin D analogue at the

**Fig. 2** Effect of BXL-628 ( $1 \times 10^{-8}$  M) on Matrigel invasion of DU145 cells (panel a) and PC3 cells (panel b) in basal conditions and following stimulation with KGF (10 ng/ml). Matrigel invasion was evaluated by using Boyden chambers. Number of cells migrated was evaluated in at least ten fields for each experimental point and averaged. Data are mean  $\pm$  SEM of the percentage of cell migrated with respect to control of four different experiments





**Fig. 3** Effect of BXL-628 ( $1 \times 10^{-8}$  M) on KGF (10 ng/ml)-induced autotransphosphorylation of its receptor. Cells were pre-treated (panel b) or not (panel a) for 4 h with  $\alpha$ -amanitin (4  $\mu$ g/ml, 4 h). After stimulations, cell lysates were immunoprecipitated using anti-KGFR antibody, run onto SDS-PAGE and analyzed first for expression of phosphorylation using anti-phosphotyrosine (PY20) antibody (*upper blots*) and, after stripping and re-probing, for receptor expression using anti-KGFR and, for the experiment with  $\alpha$ -amanitin, for actin expression. Representative of two similar experiments

concentration of  $1 \times 10^{-8}$  M. Similar results were observed in the PC3 cell line (Fig. 2, panel b).

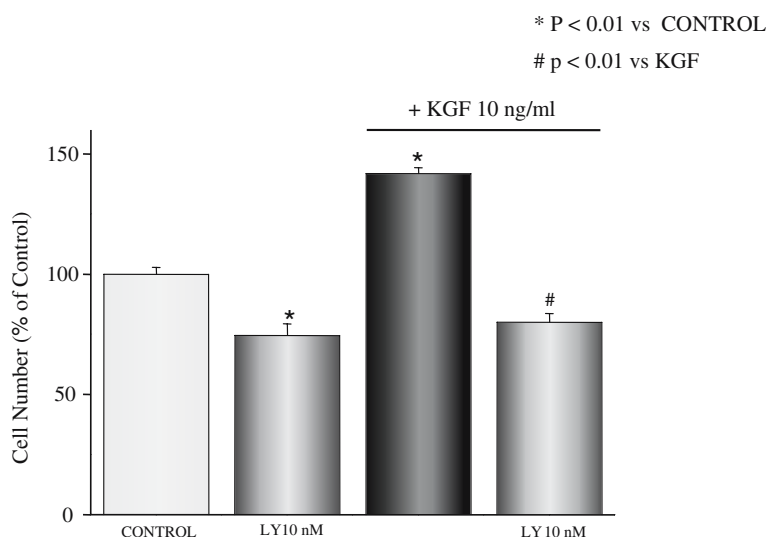
#### Effects of BXL-628 on KGF-induced signaling pathways

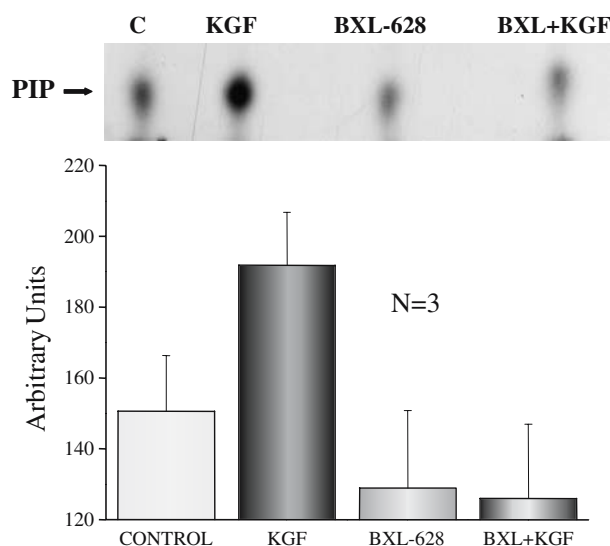
Next, we sought to investigate the effects of the compound on KGF-induced signaling in DU145 cells. In

particular, we evaluated the effect of the analogue on KGFR autotransphosphorylation and on the downstream signaling pathway PI3K/AKT. Cells were pre-treated for a short time (5 min) with BXL-628 before addition of KGF. For autotransphosphorylation studies, KGFR receptor was immunoprecipitated using specific antibody. As shown in Fig. 3a, BXL-628 abrogated KGF-induced autotransphosphorylation of its receptor. Interestingly, this effect was obtained following a brief (5 min) preincubation with the analogue, suggesting a rapid, nongenomic effect. To further investigate this possibility, we used the RNA transcription inhibitor  $\alpha$ -amanitin. As shown in Fig. 3b, the inhibitory effect of BXL-628 was still present when the experiment was conducted following 4-h incubation with 4  $\mu$ g/ml  $\alpha$ -amanitin, strongly indicating absence of transcriptional regulation in the inhibitory effect of BXL-628 on KGFR autophosphorylation.

In view of the key role exerted by the PI3K/AKT signaling pathway on invasion and migration of PC3 cells (Bonaccorsi et al. 2004a) as well as on KGF-mediated proliferation of DU145 cells (Fig. 4) where the inhibitory effect of the PI3K inhibitor LY294002 on KGF-induced proliferation of DU145 cells is shown, we evaluated the effect of BXL-628 on KGF-induced PI3K activation. As shown in Fig. 5, BXL-628 inhibits the stimulatory effect of KGF on PI3K activity. Next, we evaluated the effect of the compound on the main downstream PI3K effector, the serine/threonine kinase AKT (Wymann and Pirola 1998). AKT is activated by serine/threonine phosphorylation following PI3K activation and this phosphorylation is essential for its activity. When AKT is activated, it regulates a variety of cellular functions including cell survival, cell growth, cell differentiation, cell cycle progression, and cell metabolism (Paez and Sellers 2003). Thus, we evaluated serine phosphorylation of AKT by Western blot analysis, employing a specific anti-serine phosphorylated AKT antibody, following DU145 cell stimulation with KGF

**Fig. 4** Effect of the phosphatidylinositol-3 kinase inhibitor, LY294002, on proliferation of DU145 cells, determined by cell counting. Cells were treated for 48 h with fixed concentration of LY294002 (10 nM) with or without KGF (10 ng/ml). Each experimental point was determined in triplicate and experiments were performed at least three times. Results are expressed as the percentage of growth compared with their relative controls



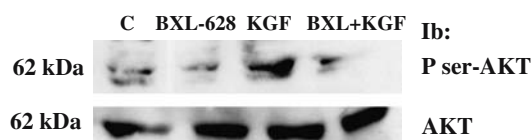


**Fig. 5** Effect of BXL-628 ( $1 \times 10^{-8}$  M) on KGF (10 ng/ml)-mediated PI3K activation. After stimulation, cell lysates were immunoprecipitated using an anti-phosphotyrosine (PY20) antibody, followed by immunokinase assay in the presence of [ $\gamma$ - $^{32}$ P]ATP (for details, see [Materials and methods](#)). Products of the reaction are evaluated by thin-layer chromatography followed by autoradiography. *Upper panels* show representative experiments, where spots correspond to the PI3-kinase catalytic product [ $^{32}$ P]phosphatidylinositol phosphate (PIP), while *lower panels* show mean  $\pm$  SEM quantification (arbitrary units) of the band for the indicated number of experiments

in the presence or absence of BXL-628. BXL-628 inhibits KGF-stimulated AKT serine phosphorylation (Fig. 6) in agreement with the results on receptor autophosphorylation (Fig. 3) and PI3K activity (Fig. 5).

## Discussion

Prostate cancer in advanced stages is a fatal disease because of failure of androgen deprivation therapy and lack of alternative effective therapy. An ideal therapeutic agent for AI-PC should target both proliferation as well as invasive and metastatic properties of the tumor cells, since once progressed to androgen independence, PC is characterized also by higher invasive ability (Chung et al. 2005). The present study demonstrates, for the first time, that the vitamin D analogue BXL-628 is able to



**Fig. 6** Effect of BXL-628 ( $1 \times 10^{-8}$  M) on KGF (10 ng/ml)-mediated phosphorylation of the PI3K downstream effector AKT. After stimulation, equal amount of total cell lysates was subjected to SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-phosphoserine AKT antibodies (*upper panels*) followed by stripping and re-probing with anti-AKT antibodies (*lower panels*). Representative of two similar experiments

reduce both proliferation and invasive ability of the AI-PC cell lines, DU145 and PC3, in basal conditions and in response to a main growth factor, namely KGF, implicated in proliferation, progression, and invasion of PC (Russell et al. 1998). At least in part, the antiproliferative effect of BXL-628 is due to induction of apoptosis, as demonstrated by increased surface exposure of PS in live cells after treatment with the analogue. This result is in line with previous data from our group showing induction of apoptosis by vitamin D analogues in several cell types (Crescioli et al. 2000, 2002, 2003, 2004).

Keratinocyte growth factor (KGF) is a physiological paracrine factor for prostate epithelial cells produced by stromal cells under the control of androgen (Planz et al. 1999a), but in the case of PC the paracrine loop is mostly replaced by an autocrine one (Planz et al. 1999b) with enhanced effectiveness on cell proliferation. Thus, the growth factor and its receptor represent an important target for therapeutic strategies in advanced PC. Several receptor tyrosine kinase (RTK) inhibitors have been developed in recent years to specifically block RTKs such as EGFR, VGFR, and FGFR (Noble et al. 2004). Among these, Gefitinib, an inhibitor of EGFR tyrosine kinase, has been shown to effectively block, in vitro, EGFR signaling and EGF-mediated proliferation and invasion of PC cell lines (Vicentini et al. 2003; Bonaccorsi et al. 2004b). However, despite clear effectiveness in other solid tumors (Blay et al. 2005), results of a phase II clinical trial for PC with this inhibitor were disappointing (Canil et al. 2005). Lack of effectiveness of Gefitinib has been demonstrated also for renal and bladder cancers (Drucker et al. 2003; Petrylak et al. 2003) although abnormal EGFR expression/signaling has been demonstrated in these malignancies, suggesting tissue selectivity for these agents. It is likely that combination with other therapies is required for the treatment of these malignancies. Here, we show that BXL-628, consistent with previous results obtained with vitamin D analogue BXL-353 (Crescioli et al. 2002), is able to inhibit, as RTK inhibitors, KGFR autophosphorylation in DU145 cells through a rapid, likely nongenomic, mechanism of action. Although further studies are needed to define the molecular mechanism underlying KGFR tyrosine phosphorylation inhibition, the demonstration that the downstream PI3K/AKT pathway is also inhibited, suggests that BXL-628 is effective in blocking KGF action. As mentioned above, BXL-628 is less hypercalcemic compared to calcitriol and other analogues. In addition, BXL-628 is currently being tested in phase II clinical trials for the treatment of BPH and preliminary results indicate significant reduction of prostate volume compared to placebo adverse effects (Montorsi F, presentation at the EUA, Istanbul, March 2005), strongly indicating that the prostate is a target for this drug.

In conclusion, our results show that the nonhypercalcemic vitamin D analogue, BXL-628, is able to block proliferation and invasion in response to KGF in the AI cell line DU145. Together with several evidence in the



literature pointing out a differentiating role of calcitriol and its analogues in carcinoma cells (Stewart and Weigel 2004), our data provide a rationale for the development of novel analogues to be employed in the treatment of AI or advanced PC. As previously suggested (Beer et al. 2005) these analogues may be used in combination with other antineoplastic agents (such as RTK inhibitors) to induce additive or synergistic effects by simultaneously targeting multiple components of the same pathway or several targets at the same time. On the other hand, targeting multiple signaling pathways appears to be the most practicable way in approaching prostate cancer (McCarty 2004).

**Acknowledgements** This work was supported by AIRC (Milan, grant to Mario Maggi), Italian Ministry of Education (Progetti Di Rilevanza Nazionale, PRIN, to Gianni Forti) and BIOSELL (Milan, Italy).

## References

- Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM, McEwan RN (1987) A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Res* 47:3239–3245
- Baldi E, Bonaccorsi L, Forti G (2003) Androgen receptor: good guy or bad guy in prostate cancer invasion? *Endocrinology* 144:1653–1655
- Beer TM, Myrthue A, Eilers KM (2005) Rationale for the development and current status of calcitriol in androgen-independent prostate cancer. *World J Urol* 23:28–32
- Blay JY, Le Cesne A, Alberti L, Ray-Coquart I (2005) Targeted cancer therapies. *Bull Cancer* 92:E13–E18
- Bonaccorsi L, Luconi M, Maggi M, Muratori M, Forti G, Serio M, Baldi E (1997) Protein tyrosine kinase, mitogen-activated protein kinase and protein kinase C are involved in the mitogenic signaling of platelet-activating factor (PAF) in HEC-1A cells. *Biochim Biophys Acta* 1355:155–166
- Bonaccorsi L, Carloni V, Muratori M, Salvadori A, Giannini A, Carini M, Serio M, Forti G, Baldi E (2000) Androgen receptor expression in prostate carcinoma cells suppresses alpha6beta4 integrin-mediated invasive phenotype. *Endocrinology* 141:3172–3182
- Bonaccorsi L, Carloni V, Muratori M, Formigli L, Zecchi S, Forti G, Baldi E (2004a) EGF receptor (EGFR) signaling promoting invasion is disrupted in androgen-sensitive prostate cancer cells by an interaction between EGFR and androgen receptor (AR). *Int J Cancer* 112:78–86
- Bonaccorsi L, Marchiani S, Muratori M, Forti G, Baldi E (2004b) Gefitinib ('IRESSA', ZD1839) inhibits EGF-induced invasion in prostate cancer cells by suppressing PI3 K/AKT activation. *J Cancer Res Clin Oncol* 130:604–614
- Canil CM, Moore MJ, Winquist E, Baetz T, Pollak M, Chi KN, Berry S, Ernst DS, Douglas L, Brundage M, Fisher B, McKenna A, Seymour L (2005) Randomized phase II study of two doses of gefitinib in hormone-refractory prostate cancer: a trial of the National Cancer Institute of Canada-Clinical Trials Group. *J Clin Oncol* 23:455–460
- Chung LW, Baseman A, Assikis V, Zhau HE (2005) Molecular insights into prostate cancer progression: the missing link of tumor microenvironment. *J Urol* 173:10–20
- Crescioli C, Maggi M, Vannelli GB, Luconi M, Salerno R, Barni T, Gulisano M, Forti G, Serio M (2000) Effect of a vitamin D3 analogue on keratinocyte growth factor-induced cell proliferation in benign prostate hyperplasia. *J Clin Endocrinol Metab* 85:2576–2583
- Crescioli C, Maggi M, Luconi M, Vannelli GB, Salerno R, Sinisi AA, Bonaccorsi L, Ferruzzi P, Barni T, Forti G, Serio M (2002) Vitamin D3 analogue inhibits keratinocyte growth factor signaling and induces apoptosis in human prostate cancer cells. *Prostate* 50:15–26
- Crescioli C, Ferruzzi P, Caporali A, Mancina R, Comerci A, Muratori M, Scaltriti M, Vannelli GB, Smirardo S, Mariani R, Villari D, Bettuzzi S, Serio M, Adorini L, Maggi M (2003) Inhibition of spontaneous and androgen-induced prostate growth by a nonhypercalcemic calcitriol analog. *Endocrinology* 144:3046–3057
- Crescioli C, Ferruzzi P, Caporali A, Scaltriti M, Bettuzzi S, Mancina R, Gelmini S, Serio M, Villari D, Vannelli GB, Colli E, Adorini L, Maggi M (2004) Inhibition of prostate cell growth by BXL-628, a calcitriol analogue selected for a phase II clinical trial in patients with benign prostate hyperplasia. *Eur J Endocrinol* 150:591–603
- Drucker B, Bacik J, Ginsberg M, Marion S, Russo P, Mazumdar M, Motzer R (2003) Phase II trial of ZD1839 (IRESSA) in patients with advanced renal cell carcinoma. *Invest New Drugs* 21:341–345
- Gronberg H (2003) Prostate cancer epidemiology. *Lancet* 361:859–864
- Habuchi T, Suzuki T, Sasaki R, Wang L, Sato K, Satoh S, Akao T, Tsuchiya N, Shimoda N, Wada Y, Koizumi A, Chihara J, Ogawa O, Kato T (2000) Association of vitamin D receptor gene polymorphism with prostate cancer and benign prostatic hyperplasia in a Japanese population. *Cancer Res* 60:305–308
- Kagan VE, Fabisiak JP, Shvedova AA, Tyurina YY, Tyurin VA, Schor NF, Kawai K (2000) Oxidative signaling pathway for externalization of plasma membrane phosphatidylserine during apoptosis. *FEBS Lett* 477:1–7
- Koli K, Keski-Oja J (2000) 1alpha,25-dihydroxyvitamin D3 and its analogues down-regulate cell invasion-associated proteases in cultured malignant cells. *Cell Growth Differ* 11:221–229
- Luconi M, Carloni V, Marra F, Ferruzzi P, Forti G, Baldi E (2004) Increased phosphorylation of AKAP by inhibition of phosphatidylinositol 3-kinase enhances human sperm motility through tail recruitment of protein kinase A. *J Cell Sci* 117:1235–1246
- McCarty MF (2004) Targeting multiple signaling pathways as a strategy for managing prostate cancer: multifocal signal modulation therapy. *Integr Cancer Ther* 3:349–380
- Nagpal S, Na S, Rathnachalam R (2005) Noncalcemic actions of vitamin D receptor ligands. *Endocr Rev* 26(5):662–687
- Noble ME, Endicott JA, Johnson LN (2004) Protein kinase inhibitors: insights into drug design from structure. *Science* 303:1800–1805
- Paez J, Sellers WR (2003) PI3K/PTEN/AKT pathway. A critical mediator of oncogenic signaling. *Cancer Treat Res* 115:145–167
- Peehl DM, Feldman D (2004) Interaction of nuclear receptor ligands with the Vitamin D signaling pathway in prostate cancer. *J Steroid Biochem Mol Biol* 92:307–315
- Petrylak D, Faulkner JR, Van Veldhuizen PJ et al (2003) Evaluation of ZD1839 for advanced transitional cell carcinoma (TCC) of the urothelium: a Southwest Oncology Group Trial (abstract 1619). *Proc Am Soc Clin Oncol* 22:403
- Planz B, Aretz HT, Wang Q, Tabatabaei S, Kirley SD, Lin CW, McDougal WS (1999a) Immunolocalization of the keratinocyte growth factor in benign and neoplastic human prostate and its relation to androgen receptor. *Prostate* 41:233–242
- Planz B, Kirley SD, Wang Q, Tabatabaei S, Aretz HT, McDougal WS (1999b) Characterization of a stromal cell model of the human benign and malignant prostate from explant culture. *J Urol* 161:1329–1336
- Russell PJ, Bennett S, Stricker P (1998) Growth factor involvement in progression of prostate cancer. *Clin Chem* 44:705–723
- Schwartz GG, Wang MH, Zang M, Singh RK, Siegal GP (1997) 1 alpha,25-Dihydroxyvitamin D (calcitriol) inhibits the invasiveness of human prostate cancer cells. *Cancer Epidemiol Biomarkers Prev* 6:727–732

- So A, Gleave M, Hurtado-Col A, Nelson C (2005) Mechanisms of the development of androgen independence in prostate cancer. *World J Urol* 23:1–9
- Stewart LV, Weigel NL (2004) Vitamin D and prostate cancer. *Exp Biol Med* (Maywood) 229:277–284
- Sung V, Feldman D (2000) 1,25-Dihydroxyvitamin D3 decreases human prostate cancer cell adhesion and migration. *Mol Cell Endocrinol* 164:133–143
- Trump DL, Hershberger PA, Bernardi RJ, Ahmed S, Muindi J, Fakih M, Yu WD, Johnson CS (2004) Anti-tumor activity of calcitriol: pre-clinical and clinical studies. *J Steroid Biochem Mol Biol* 89–90:519–526
- Vicentini C, Festuccia C, Gravina GL, Angelucci A, Marronaro A, Bologna M (2003) Prostate cancer cell proliferation is strongly reduced by the epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 in vitro on human cell lines and primary cultures. *J Cancer Res Clin Oncol* 129:165–174
- Wymann MP, Pirola L (1998) Structure and function of phosphoinositide 3-kinases. *Biochim Biophys Acta* 1436:127–150
- Yudoh K, Matsuno H, Kimura T (1999) 1 $\alpha$ ,25-dihydroxyvitamin D3 inhibits in vitro invasiveness through the extracellular matrix and in vivo pulmonary metastasis of B16 mouse melanoma. *J Lab Clin Med* 133:120–128